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 Agrobacterium sp. ATCC 55002 - useful for degrading ferric
 chelates of amino poly:carboxylic acid(s)
 C91-140556 R(AT BE CH DE DK ES FR GB GR IT LU NL SE) N(CA
 JP)

Biologically pure culture of Agrobacterium sp ATCC 55002
 is new.

Also claimed is a method of degrading ferric chelates (I)
 of aminopolycarboxylic acids (II) in aq. solns. by combining
 (I) with the above culture.

USE/ADVANTAGE

The method is rapid and does not damage the environm-
 ent, and may be used to treat a range of industrial wastes.

PREFERRED PROCESS

(I) are degraded in aq. soln. at a pH <8 (esp. adjusted
 with inorg. mineral acid) in the presence of other C sources
 (esp. sucrose) and/or N sources (esp. yeast extract), pref.
 with added basal medium and buffer (esp. a phosphate buf-
 fer).

(I) are esp. ferric chelates of EDTA and/or propylene-

D(4-A1J, 4-B5, 4-B6, 5-H4) E(5-L2A, 11-M, 11-Q2)

diaminetetraacetic acid. In the process, Fe pptes. from
 soln.

MICROORGANISM

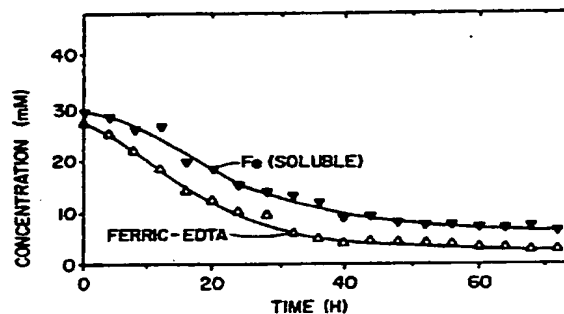
Sp. ATCC 55002 was isolated from sludge in an industr-
 ial waste treatment facility receiving ferric EDTA complex.

EXAMPLE

Agrobacterium sp ATCC 55002 was cultured on an aq.
 medium contg. ferric EDTA and basal medium at 28°C.

The fig. shows the relative concns. of sol. Fe and
 ferric EDTA during the incubation, using ferric EDTA as
 the sole carbon source. (38pp478DAHDwgNo3b/6).
 (E) ISR: J58043782 US4673644 US4634667.2.Jnl.Ref.

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(54) Title: DEGRADATION OF FERRIC CHELATES BY A PURE CULTURE OF <i>AGROBACTERIUM SP.</i>		
(57) Abstract A novel microorganism has been isolated and identified as <i>Agrobacterium sp.</i> , a gram-negative aerobe. The <i>Agrobacterium sp.</i> microorganism has been found to efficiently degrade ferric chelates of aminopolycarboxylic acids, such as ethylenediaminetetraacetic acid and related compounds, found in aqueous waste solutions. A biologically pure culture of <i>Agrobacterium sp.</i> and a process for using the culture are disclosed.		

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DEGRADATION OF FERRIC CHELATES BY A PURE
CULTURE OF AGROBACTERIUM SP.

5

BACKGROUND OF THE INVENTION

1. Field of the Invention.

The present invention relates to biological degradation of ferric chelates of aminopolycarboxylic acids. More particularly, the invention relates to the use of the
10 microorganism Agrobacterium sp. to degrade these compounds, especially from aqueous solutions, as well as to biologically pure cultures of Agrobacterium sp.

2. State of the Art.

It has heretofore been recognized that metal chelates of
15 aminopolycarboxylic acids, such as ferric-ethylenediaminetetraacetic acid (ferric-EDTA) and other similar chelates, were not readily biodegraded. See, for example, Gerike et al., Ecotoxicology and Env. Safety, 3:159-173 (1975).

This inability to readily degrade such metal chelates
20 leads to numerous environmental concerns. In particular, in secondary treatment with an activated sludge, the biodegradation of these metal chelates is so slow that these chelates have been reported to pass through secondary treatment facilities and discharged into the environment without notable degradation.
25 See Thom et al., Proc. Roy. Soc. of London, 189:347-357 (1975). This has led to concerns for the possible bioaccumulation of the chelate in the environment as well as the possible transport of heavy metals by release of high concentrations into polluted rivers or sediments. Additionally, EDTA has also been
30 implicated in the mobilization of radionuclides from nuclear waste disposal sites.

On the other hand, accumulation of EDTA in the environment over time periods on the order of years has been shown to be unlikely. For example, EDTA contamination in soil, at relatively low concentrations (2-1000 micrograms per gram of soil), have been demonstrated to be biologically removed. See Tiedje, Appl. Microbiol., 30:327-329 (1975) and Tiedje, J. Environ Qual. 6:21-26 (1977). In these studies, the disappearance of low concentrations of EDTA and its metal chelates was shown to occur in 15-45 weeks on a wide variety of soils. However, no single bacterial type was isolated which was capable of metabolizing EDTA or its metal chelates.

At somewhat higher concentrations (< 2mM) in aqueous solution, Belly et al., Appl. Microbiol., 29:787-794 (1975) have investigated the aerobic bacterial degradation of the Fe⁺³ EDTA chelate by an acclimated mixed population of bacteria. Disappearance of more than 90% of the substrate in 5 days was observed at these concentrations. Again, no single bacterial type was isolated which would grow on the ferric-EDTA substrate as sole carbon source.

The abstract for SU 525627 describes removal of heavy metal complexes from water by precipitation, using first a sodium hydroxide treatment and then biological purification using aerobic microorganisms. The filtrate obtained from the sodium hydroxide treatment is purified with microorganisms at a pH of 8-9.

Japanese Laid-Open Patent Application (Kokai) No. 58-43782 states that strains of the genera Pseudomonas and Alcaligenes are capable of degrading EDTA under aerobic conditions. The maximum concentration tested was 5 mM and required 5 days for 80% degradation.

Besides biological degradation, the ferric chelate of EDTA can be degraded by ultraviolet irradiation. Specifically, under simulated environmental conditions, Lockhart et al., Env. Sci. and Technol., 9:1035-1038 (1975) as well as Lockhart et al., Environ. Lett., 9:19-31 (1975) have demonstrated

degradation by the mechanism of UV irradiation. Rates of degradation in bright sunlight were found to vary from 1-2 days for removal of the parent compound at an initial concentration and pH of 1.6 mM and 4.9, respectively, to greater than 10 days at an initial concentration and pH of 9.8 mM and 6.9, respectively. In these studies transformation of ferric-EDTA was demonstrated but extensive mineralization was not.

In view of the above, there is a continuing need in the art for an efficient method for the degradation of metal chelates of aminopolycarboxylic acids, especially from industrial waste solutions contaminated with such chelates. There is a further need in the art that any such degradation process for the removal of such chelates be conducted rapidly without damaging the environment.

15

SUMMARY OF THE INVENTION

The present invention is directed to the discovery that the microorganism Agrobacterium sp. (deposited with the American Type Culture Collection, Rockville, MD., as ATCC No. 55002) rapidly and efficiently degrades ferric chelates of aminopolycarboxylic acids and accordingly, this organism can be used to treat aqueous solutions so as to remove such ferric chelates.

Accordingly, in one of its composition aspects, the present invention is directed to a biologically pure culture of the microorganism Agrobacterium sp., ATCC number 55002.

In another of its composition aspects, the present invention is directed to a biologically pure culture of Agrobacterium sp., which culture is capable of degrading ferric chelates of aminopolycarboxylic acids from an aqueous solution containing one of more of the chelates.

In one of its method aspects, the present invention is directed to a method of degrading ferric chelates of

aminopolycarboxylic acids which comprises combining said chelates with a biologically pure culture of Agrobacterium sp. This aspect of the invention further relates to a method for degrading ferric chelates of ethylenediaminetetraacetic acid, 5 propylenediaminetetraacetic acid or mixtures thereof which comprises combining one or more of said chelates with a biologically pure culture of Agrobacterium sp.

In another method aspect, the present invention is directed to a method of recovering iron from an aqueous 10 solution containing ferric chelates of aminopolycarboxylic acid which method comprises

- a) adding a biologically pure culture of Agrobacterium sp. to an aqueous solution containing one or more ferric chelates of aminopolycarboxylic acid;
- 15 b) maintaining the aqueous solution at a pH of about 8 or less at a temperature and for a period of time sufficient for iron to precipitate from solution; and
- c) recovering said iron.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figures 1(a) and 1(b) illustrate characteristics of the growth of Agrobacterium sp. (ATCC No. 55002) in shakeflasks with sodium ferric-EDTA. Solutions containing basal medium (described below), 35 mM sodium ferric-EDTA and 100 mM phosphate were adjusted to pH 6.2 and 7.4 by the addition of HCl and NaOH 25 respectively. The incubations proceeded in shaker-incubators at 29°C. In Figure 1(a), the optical densities at 600 nm are plotted for aliquots diluted in 0.1 N HCl with the plotted values corrected for the dilution factor. In Figure 1(b), ferric-EDTA concentration is plotted in millimolar units and the 30 measured pH is given above the data points.

Figures 2(a) and 2(b) illustrate results obtained from incubation of Agrobacterium sp. (ATCC No. 55002) at the 1 liter scale with pH controlled at 7.4 and temperature maintained between 29-31°C. The initial broth was prepared to contain 35 35 mM sodium ferric-EDTA and 100 mM phosphate in basal medium. In

particular, Figure 2(a) illustrates Chemical Oxygen Demand (COD) measurements at different times during the incubation. Specifically, aliquots of the whole broth were taken for COD measurements (COD with cells) and after centrifugation at 13000 g to remove cell mass and any chemical precipitate (COD supernatant). Figure 2(b) illustrates the concentration of soluble iron (i.e., in solution), of ammonia and of ferric-EDTA at different points of the incubation.

Figures 3(a) and 3(b) illustrate results (including carbon and nitrogen mass balance) from a large scale (7.2 liter) incubation of Agrobacterium sp. (ATCC 55002) with ferric-EDTA. The COD values were obtained for aliquots after centrifugation to remove cells. The conversion of carbon and nitrogen from ferric-EDTA to CO₂ and ammonia are plotted as percentages of the maximum values possible from the initial concentration of ferric-EDTA measured at the time of inoculation. The percentage of COD removed is calculated by subtracting that remaining in solution from that in the initial broth. Neither COD nor ammonia values were corrected for evaporation.

Figure 4 illustrates the effect of initial pH and ferric-EDTA concentration on the growth of Agrobacterium sp. (ATCC 55002). Test tubes contained basal medium, phosphate buffer (100 mM) and sodium ferric-EDTA at the concentrations indicated in the Figure. Test tubes were inoculated at a calculated initial Optical Density (OD) of 1.0 from a concentrate of freshly harvested and washed cells and were incubated at 30°C ± 1°C for 48 hours. Error bars indicate the standard deviation for triplicate experiments (pH 7.4) and duplicate experiments (pH 6.2). Controls were not run in replicate.

Figure 5 is an electron micrograph of Agrobacterium sp., platinum/palladium shadowed, grown on tryptic soy broth agar plates.

Figure 6 is an electron micrograph of Agrobacterium sp., platinum/palladium shadowed, grown on ferric-EDTA agar plates.

The small dark patches indicate localized high-density regions on the surface of the bacterium.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A biologically pure culture of Agrobacterium sp., ATCC
5 No. 55002, was isolated from a treatment facility receiving
industrial waste containing the ferric chelate of
ethylenediaminetetraacetic acid (ferric-EDTA) and is capable of
degrading ferric chelates of aminopolycarboxylic acids at rates
and substrate concentrations significantly greater than any
10 previously reported. This microorganism is an aerobic, gram
negative rod of approximately 1 micron in diameter and
approximately 2-3 microns in length. For purposes of this
application, a biologically pure culture of Agrobacterium sp. is
a culture which contains cells of one kind, all progeny of a
15 single ancestor or identical ancestors as well as mutants and
variants thereof having the distinguishing features of
Agrobacterium sp.

It has been discovered that the isolated Agrobacterium
sp. microorganism metabolizes ferric-EDTA as the sole carbon
20 source with a rate of consumption as rapid as 24 mM/day.
Preferably, in aqueous solutions, the ferric-EDTA concentrations
can range from about 1 mM or less up to about 150 mM or more and
more preferably from about 3 mM up to about 80 mM. Thus, the
microorganism of the invention is capable of degrading ferric-
25 EDTA at a rapid rate and at high substrate concentrations. As
metabolism proceeds, carbon dioxide and ammonia are produced, pH
rises, and iron is precipitated. The precipitated iron can be
recovered by any conventional means such as filtration,
centrifugation, etc. The isolated microorganism, Agrobacterium
30 sp., also metabolizes the ferric chelates of other
aminopolycarboxylic acids, such as propylene diaminetetraacetic
acid (PDTA) and the like.

For purposes of this application, the term "ferric
35 chelates of aminopolycarboxylic acid" means those
aminopolycarboxylic acids having two or more amino group and 3

or more carboxylic acid groups and which are capable of forming chelates with Fe^{+3} . Aminopoly- carboxylic acids which may be degraded (as the ferric chelate) by the microorganism of the invention include, for example, ethylenediaminetetraacetic acid
5 (EDTA), propylenediaminetetraacetic acid (PDTA), and the like.

A biologically pure culture of the newly discovered microorganism, Agrobacterium sp., can be used in a process for the removal of ferric chelates of aminopolycarboxylic acids. Preferably, the process is conducted in an aqueous solution
10 wherein the aqueous solution is maintained at a pH of about 8.0 or less. Alternatively, it is contemplated that the process can be conducted in a landfill, or other similar site, which has been contaminated with ferric chelates of aminopolycarboxylic acids. As before, it is preferable for the pH of the medium to
15 adjusted to a pH of about 8.0 or less.

When conducted in an aqueous solution, it is contemplated that the process is useful with a wide variety of aqueous solutions such as industrial waste solutions, cleaning solutions, among others. Additionally, the aqueous solution
20 employed herein should not contain biocidal effective amounts of a biocide. In a preferred embodiment, in order to enhance the degradation rate of ferric chelates, the aqueous solution should contain less than an inhibitory effective amounts of inhibitors of Agrobacterium sp., i.e., an amount of an inhibitory agent
25 which causes a 50% decrease in ferric-aminopolycarboxylic acid metabolism.

In preferred embodiments of the process of the invention a mineral and biotin stock is added to the aqueous solution. Specifically, the salts ammonium chloride, magnesium
30 sulfate and calcium sulfate can be used at concentrations of 5 mM, 0.1 mM and 0.07 mM respectively. In these embodiments, certain trace mineral salts are added to the aqueous solution from which the ferric chelates are to be degraded in order to provide an improved media for growth of the microorganisms.
35 These salts may include H_3BO_3 , $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot \text{H}_2\text{O}$, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$, $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$. The trace salts

preferably are present in amounts of less than about 25 micromolar. Specifically, these salts can be employed at concentrations of 0.025mM, 0.003 mM, 0.002 mM, 0.00035 mM, 0.0002 mM and 0.00004 mM respectively. Biotin may also be added
5 to the solution and typically is present in amounts of about 1 to about 10 micromolar. Specifically, biotin can be employed at a concentration of 2 micromolar. Aqueous solutions containing the specific concentrations recited above of the salts, trace mineral salts, and biotin are sometimes referred to herein as
10 "basal medium".

Other ingredients which may be present in the aqueous solution include compatible buffers, i.e., buffers which are not toxic to the microorganism, such as sodium phosphate, potassium phosphate, and the like. A sufficient amount of buffer is added
15 so as to maintain the pH of the aqueous solution in the desired range. The preferred buffer for the process of the invention is phosphate buffer, e.g., a 50/50 molar mix of sodium and potassium salts at a total phosphate concentration of from 25 to 200 mM. However, any compatible buffer or buffers can be used
20 if acid is periodically added to maintain the pH at a level of below about 8.0. Preferably, the pH of the aqueous solution is maintained between about 6 and 8 and preferably, the buffer or buffers are added to the aqueous solution generally are present in amounts of about 25 mM to about 200 mM.

25 In order to maintain the aqueous solution at a pH of about 8.0 or less, acid may be added to the solution. The acid can be any acid which does not affect the degradation of the ferric chelates by the Agrobacterium sp. microorganism. Such acids include, HCl, H₂SO₄, and the like. Preferably, HCl is
30 used. The acid is added periodically to keep the pH below about 8.0.

The temperature of the aqueous solution containing both the metal chelate and Agrobacterium sp. is not critical provided that a temperature is employed which is compatible with
35 the microorganism. Accordingly, any temperature may be employed which is sufficient to allow the microorganism to degrade ferric

chelates. In preferred embodiment, the temperatures employed range from about 21°C to about 37°C and even more preferably, from about 29°C to about 31°C.

The following examples illustrate the isolation, identification and testing of Agrobacterium sp. and are provided to further illustrate the invention, but are not meant to limit the scope of the claims in any way.

EXAMPLES

The procedures described below were used to evaluate the microorganism of the invention.

Bacterial isolations. Samples of sludge were obtained from an aerated secondary waste treatment facility that had been receiving wastes containing ferric-EDTA for several years. Samples were collected in plastic bottles, transported on ice and then held at 4°C prior to use. An enrichment medium (EDTA EM, See Table 1) was inoculated with 200 microliter of sample and incubated at 30°C for two weeks. Growth from this initial enrichment was streaked onto agar plate medium (agar solidified EDTA EM) and incubated at 30°C until growth was observed. Bacterial colonies from these plates were repeatedly transferred and tested for growth on a stringent agar medium (EDTA M1 and M2, see Table 1) to select for colonies using ferric-EDTA as sole carbon source. Isolated colonies on these media were then transferred to aerobic liquid culture media containing ferric-EDTA as sole carbon source (EDTA G1, see Table 1).

Table 1

Enrichment, Isolation and Growth Media^a

<u>Ingredient</u>	<u>Concentration (mM) in Media</u>			
	<u>EDTA EM</u>	<u>EDTA M1</u>	<u>EDTA M2</u>	<u>EDTA G1</u>
5 Citrate-phosphate buffer	50 (pH 5.5)			
MES ^b		50 (pH 6)		
Phosphate buffer			50 (pH6)	100 (pH7.4)
10 Na ₂ S ₂ O ₃ · 5H ₂ O	40	40	40	
Ferric-EDTA	5 ^c	5 ^c	5 ^c	35 ^d
NaHCO ₃	24			
Agar	17 g/L ^e	17 g/L	17 g/L	

- 15 a All media employed the mineral and biotin base described at page 10 hereinabove as the basal medium.
- b [N-Morpholino]ethanesulfonic Acid
- c Ammonium salt.
- 20 d Sodium salt.
- e No agar in liquid enrichment medium EDTA EM

In all studies, media containing ferric-EDTA were sterilized prior to incubation with Agrobacterium sp.

25 In some cases, this was done by filtration through 0.2 micron filters after addition of all components and after adjustment of pH. In other cases, the total broth was not filtered, rather stock solutions were filter sterilized individually and then added together

30 at which time the pH was adjusted using an ethanol-washed pH electrode to maintain sterility. In still other cases, the stock solutions, with the exception of biotin, were heat sterilized in situ. Then a stock solution of FeCl₃ (0.1 M) was prepared for

35 supplementation of iron at 1 mM. The biotin and FeCl₃ stocks were filter sterilized and added separately. Media which was inadvertently contaminated with organism(s) other than Agrobacterium sp. did not support extensive degradation of ferric-EDTA and,

40 accordingly, media containing such other organism(s) are not preferred.

Cell Harvest and Storage. After growth in ferric-EDTA media, cells were harvested by centrifugation at 4°C. Broth was first centrifuged at 4200 g for 20 minutes and then the supernatant fluid was decanted and
5 centrifuged at 13000 g for 20 minutes. The cell pellet was resuspended in sterile distilled water and centrifuged again at 13000 g to wash the cells. The precipitated cell pellet was resuspended in sterile
10 distilled water at an optical density >5 at 600 nm. For long term storage, glycerol was added (10% by volume) to the suspension and aliquots (2 ml) stored in a liquid nitrogen freezer.

The procedure given above for washing the inoculum before storage or inoculation of fresh media was
15 adopted to produce conditions which would lead to rapid degradation. In several instances when unwashed inocula were used, growth in fresh media either lagged or was considerably slower than that normally observed. Washing removes much of the precipitate as determined
20 by light microscopy.

Alternatively, the cells can be grown in a complex trypticase/soy broth (from BBL) maintained at 29-31°C for 24 hours. The cells so grown are still effective in degrading ferric-EDTA when added to an aqueous
25 solution containing ferric-EDTA.

For inoculation, cells from frozen stocks were added or cells were harvested from seed flasks in the later stages of growth. When freshly harvested, cells were prepared as above except that after the initial
30 centrifugation at 13000 g, the cells were suspended in water or fresh media as the inoculation medium.

Electron Microscopy. Cultures were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 minutes and then dropped onto Formvar coated grids, dried and
35 vacuum coated with platinum/palladium at an angle of 14

degrees. Photomicrographs were obtained with rotary shadowing on a JEOL - 100 CX electron microscope.

5 Strain Identification. Representative samples were subcultured on blood agar and McConkey agar plates. After incubation, samples were prepared for identification by Vitek AMS using the GNI Card (Vitek Systems, Inc., Hazelwood, MO 63042) and API 20E using the API Rapid NFT (API Analytab Products, Plainview, NY 11803) systems for the identification of bacteria by 10 the analysis of biochemical reactions. In addition, gas chromatographic analysis of whole cell fatty acids as described in Drucker, Methods in Microbiology, Vol. 9, J.R. Norris, Ed. Academic Press, London, p. 51-125, was also used to confirm the identification.

15 Ferric-EDTA Assay. Cells were separated from samples prior to the determination of ferric-EDTA by centrifugation in an Eppendorf Model 5415 centrifuge at 14,000 rpm for 2 minutes. Samples were then diluted with water or initial mobile phase such that the 20 maximum ferric-EDTA concentration of the diluted samples was less than 0.9 mM.

25 HPLC analysis were performed using a modular Waters Associates system (Millipore Corp., Milford, MA 01757) equipped with a WISP 712 sample processor for injection of samples, two Waters 510 pumps for mobile phase delivery, a Waters 490 multiwavelength detector set at 360 nm for detection of the ferric-chelate and a Waters 840 data system for display, storage, plotting and analysis of the chromatograms.

30 The chromatographic separations were obtained after injection of 30 microliters of sample onto a commercially available, 15 cm long by 4.6 mm i.d. reversed-phase HPLC column (LC18 from Supelco, Inc., Bellefonte, Penn. 16823). Isocratic elution at 1.0 35 milliliter/minute with an initial mobile phase

consisting of 1 g/liter ammonium acetate, 0.5 g/liter PDTA, 1.5 milliliter/liter glacial acetic acid, 0.5 milliliter/liter ammonium hydroxide, 0.1 milliliter/liter triethylamine and 5 milliliter/liter acetonitrile in deionized water caused elution of ferric-EDTA at 2 minute retention time. After 2.5 minutes from the injection, a step-gradient to a final mobile phase consisting of 0.5 g/liter PDTA, 0.1 milliliter/liter triethylamine and 600 milliliter/liter acetonitrile in deionized water for 5.5 minutes washed highly retained components from the column. A further step gradient to the initial mobile phase for 4 minutes was used to reequilibrate the column.

The instrument was calibrated daily by the injection of a series of standards of ammonium ferric-EDTA in unbuffered, deionized water encompassing the concentrations from 0.03 to 0.9 mM. Linear-least squares regression analysis of the area responses of the standards was used to obtain slope and intercept values (r -squared typically >0.996) which were then used to obtain quantitative values for samples from the measured area. Precision and accuracy were estimated to be better than 5% relative over the calibration range.

Ammonia/Ammonium Assay. Total ammonia was determined using an Orion Model 95-12 ammonia electrode (following the procedures set forth in the Orion Model 95-12 instruction manual, Orion Research Inc.).

COD Assay. For the estimation of COD in samples, the test tube colorimetric procedure of the Hach Chemical Co. (Hach Co., Loveland, Col. 80539), which is similar to Method 508C in "Standard Methods for the Examination of Water and Wastewater" American Public Health Association, Washington, D.C., 16th Edition, pp. 532-538 (1985) was used.

Iron Assay. For the estimation of total iron in aqueous samples, a modification of Stookey's ferrozine procedure described in Stookey, Anal. Chem., 42:779-781 (1970) was used. To release the iron cation from chelators present, including ferric-EDTA, samples were first digested using a procedure adapted from Method 302E in "Standard Methods for the Examination of Water and Wastewater", American Public Health Association, Washington, D.C., 16th ed. p. 148 (1985). A 50-microliter sample was added to an acid-cleaned, thick-walled, screw-capped test tube (equivalent to Hach COD test tubes), followed by 50 microliters of concentrated nitric acid and 100 microliters of concentrated sulfuric acid. The test tube was tightly-capped and heated to reflux at 150°C for 15 minutes in a Hach Reactor Plate. After cooling, the tubes were uncapped and replaced in the reactor for a maximum of 10 minutes, but not long enough for the test tube to become thoroughly dry. After cooling, 200 microliters of acid-ferrozine reagent (0.514 g ferric, 10 g hydroxylamine hydrochloride, and 50 milliliters of concentrated hydrochloric acid dissolved and diluted to 100 ml with distilled water) was added. The contents were mixed using a vortex mixer and then 1.5 milliliters of buffer (40 g ammonium acetate and 35 milliliters of concentrated ammonium hydroxide diluted to 100 milliliters with distilled water) was added to adjust the pH between 5 and 9. The sample was then diluted with 5.4 milliliters of distilled water.

Standards encompassing the concentration range from 20 to 1000 mg/L total iron were prepared from pure ferric ammonium sulfate dodecylhydrate. Standards were treated as the samples were. From the absorbance of the standards measured at 562 nm in a spectrophotometer (Hach DR/3) and concentrations of the standards, a linear least squares calibration was constructed. At concentrations of total iron greater than 50 mg/L, the

observed precision and accuracy using samples containing known concentrations of ferric-EDTA were better than 5%.

Example 1 -- Bacterial Identification

5 The identification of the isolated strain as Agrobacterium sp., a gram-negative aerobe, was made on a Vitek AMS (Vitek Systems, Hazelwood, Missouri 63042-2395) using a Vitek GNI Card (gram negative identification card; product no. 51-1306) with a probability of 99%. The biochemical tests employed for the identification and the results for the isolated strain are presented in Table 2. The identification was also confirmed by an API Rapid NFT strip (API Analytab Products, Plainview, New York 11803; product no. 8886-500951) with a probability of 99.9%. The biochemical tests employed for the identification and the results for the isolated strain are presented in Table 3. Gas-chromatographic analysis of the fatty acid composition of the strain also confirmed the genera as Agrobacterium.

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15

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Table 2

Vitek GNI Test Results for Isolated Strain

	<u>Biochemical Test:</u>	<u>Reaction</u>	<u>Biochemical Test:</u>	
5	<u>Reaction</u>			
	Dp-300	-	Sorbitol	-
	Glucose	+	Sucrose	-
	Acetamide	-	Inositol	-
	Esculin	+	Adonitol	-
10	Plant Indican	+	p-Coumaric	-
	Urea	+	H ₂ S	-
	Citrate	-	ONPG	-
	Malonate	-	Rhamnose	-
	Tryptophan	-	Arabinose	+
15	Polymyxin B	-	Glucose (ferment.)	-
	Lactose	+	Arginine	-
	Maltose	+	Lysine	-
	Mannitol	+	Ornithine	-
	Xylose	+	Oxidase	+
	Raffinose	-		

Table 3

API Rapid NFT Test Results for Isolated Strain

	<u>Carbon Source:</u> <u>Reaction</u>	<u>Growth</u>	<u>Biochemical Test:</u>	
5	D-Glucose	+	Nitrate Reduction	+
	L-Arabinose	+	Tryptophanase	-
	D-Mannose	+	Glucose fermentation	-
	D-Mannitol	+	Arginine dihydrolase	-
	N-acetyl-D-glucosamine+		Urea	-
10	Maltose	+	Esculin	+
	D-Gluconate	+	Gelatinase	-
	Caprate	-	Beta-galactosidase	+
	Adipate	-		
	Malate	+		
15	Citrate	-		
	Phenylacetate	-		
	Oxidase	+		

In view of the above results, the microorganism was tentatively determined to be Agrobacterium radiobacter.
 20 In addition, the isolate was typed independently (American Type Culture Collection, Rockville, MD) as an Agrobacterium sp. with distinction between biovar 1 and biovar 2 not determined. Accordingly, this microorganism is referred to herein as Agrobacterium
 25 sp.

Example 2 -- Growth studies

Growth studies were carried out to determine various characteristics of the microorganism. For these studies, the basal medium recited at page 9
 30 hereinabove was employed as well as 100 mM phosphate buffer, and 35 mM sodium ferric-EDTA in Fernbach flasks. Incubation was at 29°C in a shaker-incubator. Samples were taken periodically and the pH, ferric-

EDTA concentration and optical density (OD) at 600 nm were determined. The pH was measured using an Orion combination electrode and a Corning pH meter. OD was used as a measure of bacterial growth. Measurements were made by dilution in 0.1 N HCl to dissolve the iron precipitate which formed during degradation and which interfered in reading OD due to bacterial growth.

The increase in OD observed during growth in the Fernbach flasks is shown in Figure 1(a).

In Figure 1(b), the concentration of ferric-EDTA in the flasks is plotted along with the measured pH. The plots demonstrate that as the optical density increases in all three flasks, the concentration of ferric-EDTA decreases and the pH increases. When pH does not increase, no metabolism of ferric-EDTA is observed in any of the flasks. Conversely, if the pH is not constrained from increasing above pH 8, the disappearance of ferric-EDTA stops. The apparent increase of ferric-EDTA concentration in the flask without pH adjustment is caused by evaporation after ferric-EDTA metabolism has ceased (pH > 8.1). No correction for evaporation from any of these flasks was made. The data contained in these figures demonstrates that Agrobacterium sp. grows while ferric-EDTA is being degraded and when degradation ceases, so does growth.

Example 3 -- Incubation at the 1 liter scale

The isolate was grown at the 1 liter scale in a 2 liter incubator to enable the control of pH. The initial incubator broth was 35 mM in sodium ferric-EDTA, 100 mM in phosphate, and contained the basal medium described above. In addition, FeCl₃ was added to 1 mM final concentration. For inoculation of the incubator, the strain was grown in a shakeflask at 31°C in the same media as above for 24 hours. The cells from the shakeflask were separated from the broth by centrifugation for 20 minutes at 13000 g. These were

resuspended in 200 mL of fresh broth and added to the incubator to bring the initial total volume to 1 liter.

Air was passed into the Multigen fermentor (New Brunswick Scientific Inc., Edison, N.J. 08818) through a 0.2 micron sterile filter at a flowrate of 1 liter/minutes. The fermentor (incubator) was stirred with a magnetically driven propeller shaft at 500 rpm. The hydrogen ion concentration was monitored and controlled at between pH 7.4 and 7.7 by the automatic addition of HCl. Temperature of the incubator was maintained at $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$.

The results are plotted in Figures 2(a) and 2(b). The decrease in ferric-EDTA concentration observed as the incubation proceeded was accompanied by a parallel decrease in iron concentration in solution. As the substrate was being removed from aqueous solution, the ammonium content increased. In addition, COD fell, but not in proportion to the decrease in ferric-EDTA concentration (93%). For samples measured after centrifugation to remove the cell mass and precipitate, COD fell from 8880 to 2640 mg O_2 /liter (70% decrease); for samples measured with cells and precipitate still present, COD fell from 8870 to 3392 mg O_2 /liter (62% decrease).

In addition, selected samples were diluted and plated on TSA for bacterial counting. The live bacterial count began decreasing between 46 and 70 hours after the beginning of the experiment. This coincides closely with breaks in the slopes of the curves.

Example 4 -- Inhibition

The inhibitory effect of several factors on ferric-EDTA metabolism was investigated. These experiments were carried out in test tubes using a standard set of conditions: 100 mM phosphate buffer (pH 7.4); basal medium described above; 35 mM in ferric-EDTA;

inoculation to an optical density of 1 at 600nm;
initial volume of 3.5 mL; incubation at 29-31°C for 1
to 4 days in a test tube shaker incubator.

Concentrates of the factors to be tested for inhibition
5 were added at 4 to 6 different levels to different
sterile test tubes with the rest of the volume provided
by the addition of deionized water.

The data on the inhibitory effects of several
components are summarized in Table 4. The concentra-
10 tions in the table are those which caused a 50%
decrease in metabolism of ferric-EDTA over the time of
the experiments compared to controls containing none of
the factor tested.

Table 4

15 Inhibition of Strain ATCC #55002

Conc. (mM) Causing 50% inhibition		Ferric-EDTA Metabolism Inhibition
Factor		
20 Ferric-EDTA		80
Hepes		<5
Mops		<5
Triethanolamine		15
Tris		5
25 Ionic Strength (NaCl)		540
Ammonium		130

Hepes- 4-(2-hydroxyethyl)-1-piperazineethane-
sulfonic acid

Mops-- 3-(4-Morpholino)propanesulfonic acid

30 Tris-- tris(hydroxymethyl)aminomethane

In addition to inhibition by the specific chemicals
listed above, unidentified metabolite(s) of ferric-EDTA
degradation by Agrobacterium sp. are pro-duced during
degradation and lead to product inhi-bition. Also,
35 this microorganism is inhibited by one or more
components found in photoprocessing solutions.

Example 5 -- Chelators as growth substrates

A number of metal chelators were tested as

substrates for growth. These experiments were carried out in test tubes which were placed in a benchtop shaker for 24 days at 30°C. The medium was the same as that described above for growth inhibitors except that

5 the initial phosphate buffer concentration was 71 mM and the inoculum was calculated to produce an initial optical density of 0.4. Concentrates of the organic chelators were added to the replicate test tubes at final concentrations of 4 and 20 mM. For samples

10 containing metals, a molar amount equivalent to that of the chelator was added from concentrates of FeCl_3 , NiCl_2 or CuSO_4 . After addition of all components except the inoculum, the samples were adjusted to pH 7.4 with either dilute HCl or KOH. A duplicate set of test

15 tubes was prepared as above, but with the addition to all samples of 0.25 mL of a 2.5 g/L solution of HgCl_2 to prevent bacterial growth.

Growth in the samples after 24 days was assessed by comparing the optical densities of sample tubes with

20 those in the controls with mercuric chloride added (Table 5). Those substrates which had an optical density five-fold greater than the respective controls at both concentrations tested were considered positive for growth.

Table 5
Growth Substrates for Agrobacterium sp.

	<u>Substrate</u>	<u>Growth</u>	<u>Substrate</u>	<u>Growth</u>
	EDTA	-	Fe ³⁺ EDDA	-
5	Fe ³⁺ EDTA	+	Ethylene diamine (ED)	-
	Ni ²⁺ EDTA	-	Fe ³⁺ ED	-
	Cu ²⁺ EDTA	-	TEA (triethanolamine)	-
	NTA	-	Fe ³⁺ TEA	-
	Fe ³⁺ NTA	-	Citrate	-
10	Ni ²⁺ NTA	-	Fe ³⁺ Citrate	-
	Cu ²⁺ NTA	-	Propionate	-
	PDTA	-	Fe ³⁺ Propionate	-
	Fe ³⁺ PDTA	+	Acetate	-
	Ni ²⁺ PDTA	-	Fe ³⁺ Acetate	-
15	Cu ²⁺ PDTA	-	Lysine	-
	IMDA	-	Fe ³⁺ Lysine	-
	Fe ³⁺ IMDA	-	EDDA	-

IMDA=iminodiacetic acid NTA=nitrilotriacetic acid
 EDDA=ethylenediamine diacetic acid

20 Example 6 -- Incubations at the 7.2 liter scale

In order to control aeration, mixing and pH better and to determine the carbon and nitrogen mass balance, incubations were run at volumes of 7.2 liters in computer controlled 14 liter Chemap CF 3000 incubators (Chemap AG, Volketswil, Switzerland) maintained at 28°C.

Two 6 liter batches of ferric-EDTA medium containing basal medium (described above) were inoculated with freshly harvested cells concentrated in 1.2 liters of the same basal medium. One of the incubations also received a steady feed of 250 g/L sucrose and 250 g/L yeast extract at a constant rate of 0.2 g/min. This feed composition was chosen based on previous work with the cultivation of Agrobacteria in Hofer, J. Bacteriology, 41:193-224 and Lippincott et al, The Prokaryotes, Vol. 1, Starr et al, Eds. Springer-Verlag, pp. 842-845.

Figure 3(a) is a graphical representation of the cumulative conversion of ferric-EDTA to CO₂ and to NH₃ as percentages of the maximum possible from ferric-

EDTA in the incubation with only ferric-EDTA as the carbon source. In addition, the percentage decrease in COD is also plotted. All three curves parallel each other and further demonstrate that ferric-EDTA is efficiently mineralized. Figure 3(b) plots the relative concentrations of soluble iron and ferric-EDTA during the course of the incubation supplied solely with ferric-EDTA as carbon source.

In the incubation with additional carbon and/or nitrogen sources (sucrose/yeast), degradation of ferric-EDTA and growth of the microorganism was less vigorous, although substantial degradation did occur. In particular, for the same duration of incubation, 37% of the ferric-EDTA was degraded as opposed to more than 85% for the case shown in Figure 3(b). Accordingly, substantial degradation of ferric-EDTA in waste streams containing non-biocidal carbon sources can be expected.

Example 7 -- Effect of ferric-EDTA concentrations on Degradation by Agrobacterium sp. (ATTC 55002)

To determine the maximum initial ferric-EDTA concentration that the isolate would tolerate, a series of test tube incubations using the basal medium (described above) buffered with phosphate at 100 mM were carried out for 48 hours at $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$. The initial ferric-EDTA concentration was varied from 2.9 to 140 mM. The values for the concentration removed (calculated by subtracting the concentration remaining at 48 hours from the concentration initially added) are plotted in Figure 4. No pH adjustments were made during this experiment.

The extent of degradation was related to the initial pH. At the lower initial pH, for all concentrations tested more ferric-EDTA was degraded. The degradation proceeded to consume 48 mM of substrate at an initial pH of 6.2 and 28 mM at pH 7.4. In both sets of data, the maximum degradation extended to the

highest concentrations tested.

At the higher initial hydrogen ion concentration (pH 6.2), ferric-EDTA is metabolized at a limiting rate of 24 mM/day while at the lower initial hydrogen ion concentration (pH 7.4), the maximum rate is 15 mM/day.

Example 8 -- Morphology

Cells of the isolate were grown on agar plates containing either tryptic soy broth or ferric-EDTA as sole carbon source (see Table 1, medium G1 with 17 g/L agar). Electron micrographs of several aliquots of each culture were taken as described above. Examples of these are reproduced in Figure 5 (tryptic soy broth) and Figure 6 ferric-EDTA. In both cases the culture consisted of rods from approximately 2-3 microns long by approximately 1 microns in diameter. Only on microbes grown on ferric-EDTA were small dark patches noted.

As described in the Examples, a culture of Agrobacterium sp. can be used in a process for the degradation of ferric chelates of aminopolycarboxylic acids. The process preferably employed on aqueous solutions containing such ferric chelates and is particularly suitable for degrading ferric-EDTA, ferric-PDPA, or mixtures thereof from aqueous solutions. The rate and extent of degradation are dependent on the pH, or hydrogen ion concentration. As biodegradation proceeds and such ferric chelates are consumed, both cell mass and pH increase. If the hydrogen ion concentration is allowed to fall much below 10^{-8} (pH greater than about 8), degradation and growth cease. By the addition of acid periodically to constrain pH from rising above about 8, more extensive degradation occurs.

Without being limited to any theory, in these experiments it is believed that the source of at least

some of the increase in pH can be accounted for by the production of ammonia which is observed to increase in concentration as the ferric-EDTA is metabolized. Although ammonia itself inhibits growth of the microorganism (Table 4), significant inhibition, i.e., 50% or more, appears at a higher concentration than the concentration produced in these incubations.

The degradation of the aminopolycarboxylic acids by the Agrobacterium sp. is primarily biological, as shown by the Examples. In uninoculated controls, as in those summarized in Figure 4, neither optical density nor pH significantly increased as the incubation proceeded, nor was a significant decrease of ferric-EDTA concentration observed. Conversely, in inoculated samples, as degradation proceeded, the decrease in ferric-EDTA concentration during incubation was always accompanied by an increase in optical density and pH. Finally, the rapid increase in ammonia/ammonium concentration and the substantial release of CO₂ observed in the inoculated samples demonstrates a relatively complete metabolism of the substrate. It is believed that any influence of light on the degradation was minimal or nonexistent.

The efficiency of the mineralization of organic carbon from ferric-EDTA degraded by the microorganism of the invention can be calculated from the data plotted in Figure 3(a). At 3 days, 64% of the initial carbon can be accounted for by the evolved CO₂. The COD remaining in solution at this time, after centrifugation to remove cell mass and associated precipitate, corresponds to 39% (an overestimate since the measured values for COD were not corrected for evaporation) of that initially present. Even accounting for the maximum potential errors associated with both measurements (<10% relative) and the effects of evaporation, less than 20% of the carbon initially present could have been converted to cells.

The data for the 1 liter incubations confirms the relatively small amount of cell mass produced from the biodegradation. At the end of the experiment when the ferric-EDTA concentration remaining in solution was 7% of that initially present, the difference between the COD remaining in the total broth (including cells, precipitate, and soluble metabolites) and that remaining in the solution after the cells and precipitate were removed by filtration (soluble metabolites) was less than 10% of that in the initial fresh broth (752 vs 8870 mg O₂/liter). The difference reflects the maximum oxidizable carbon which could have been incorporated into cell mass. Thus, the metabolism of ferric-EDTA by this microorganism leads primarily to mineralization and not to increased cell mass.

In addition to the CO₂ and cell mass, an unidentified degradation product or products accumulates in solution. This product(s) is evident from the residual COD present in the solution at the end of the incubation after removal of cells and precipitates. The Agrobacterium sp. (ATCC 55002) leads to degradation of 90% or more of the initial ferric-EDTA present. However, the COD reduction only approaches 70%. Thus 20-30% of the carbon initially present as ferric-EDTA remains in solution as unidentified metabolites at the end of the incubation. These metabolites can be inhibitory to the growth of the microorganism.

Although not wishing to be bound by any theory, it is believed that the microorganism of the invention has a requirement for iron. This was indicated by some preliminary growth studies. In some of these a variable and often long lag time (3 days) was observed when attempting to cultivate the strain in ferric-EDTA media which contained a large excess of EDTA. In the normal procedure for preparing media as described

above, ferric-EDTA was dissolved in a solution consisting of basal medium and phosphate buffer. After all components were dissolved, the pH of the media was adjusted. As the pH was increased, a red-orange flocculent precipitate characteristic of iron hydroxides formed. The media were then filter sterilized which removes the precipitated iron and leaves an excess of EDTA in solution. The excess becomes greater if the pH is increased further. In fact, in order to ensure minimal lag phase in the larger scale incubations 1 millimolar ferric chloride was added to the sterile-filtered incubator media to replace some of the iron removed by the filtration. This procedure consistently produced minimal lag times of less than a few hours.

Besides a deficit of iron, lag times were also found to be related to treatment of the inoculum. When an inoculum was harvested from spent ferric-EDTA media by centrifugation without washing of the cells, it was observed that, in addition to the cells, a considerable amount of chemical precipitate was present. It is believed that the precipitate is an insoluble iron salt which forms after the strong chelator, EDTA, has been degraded. In some experiments, if most of the precipitate was not removed from the inoculum by washing as noted above, slow growth was observed in subsequent incubations.

The marked preference of this microorganism for ferric-aminopolycarboxylic acids and its effectiveness at relatively high substrate concentrations makes it useful for waste remediation strategies. In uses as a cleaning, decontamination or descaling agent, a large proportion of the EDTA or other aminopolycarboxylic acid used is believed to become chelated with a heavy metal, in many cases iron. If sufficient excess iron is present in, or added to, an aqueous system to force the equilibrium of the non-iron metal chelates to the

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ferric chelate, the microorganism of the invention can be used in that environment.

Accordingly, another aspect of the present invention relates to a method for the removal of metal chelates of aminopolycarboxylic acid from an aqueous solution containing such chelates wherein the metal is any metal cation, other than Fe^{+3} , capable of chelating with said aminopolycarboxylic acid which method comprises:

- a) adding a sufficient amount of a ferric salt to the aqueous solution containing said metal chelates so that a substantial portion of said metal chelates become ferric chelates; and
- b) adding Agrobacterium sp. (ATCC 55002) to said solution while maintaining the pH of said solution at about 8 or less.

In general, sufficient amount of a ferric salt is added so that at least about 50% of the metal chelate and preferably, at least about 80% and more preferably at least about 95% is converted to ferric chelates. Suitable ferric salts for use herein include any ferric salt which is soluble in said aqueous solution, including without limitation, ferric ammonium sulfate, ferric nitrate, ferric chloride, ferric sulfate, and the like. Further, in this regard, it is noted that certain ferric salts which are insoluble in water but soluble in aqueous acidic solutions may be employed herein by merely employing an acidic aqueous medium in which such ferric salts are soluble and which the microorganism Agrobacterium sp. will tolerate.

From the foregoing description, various modifications and changes in the process will occur to those skilled in the art. All such modifications coming within the scope of the appended claims are intended to be included therein.

WHAT IS CLAIMED IS:

- 1 1. A biologically pure culture of the
2 microorganism Agrobacterium sp., ATCC number 55002.
 - 1 2. The biologically pure culture of claim 1, said
2 culture being capable of removing ferric chelates of
3 aminopolycarboxylic acids from an aqueous solution
4 containing one or more of said chelates.
 - 1 3. The biologically pure culture of claim 2,
2 wherein said aminopolycarboxylic acids are ethylene-
3 diaminetetraacetic acid, propylenediaminetetraacetic
4 acid or mixtures thereof.
 - 1 4. A method of degrading ferric chelates of
2 aminopolycarboxylic acids which comprises combining
3 said chelates with a biologically pure culture of
4 Agrobacterium sp.
 - 1 5. The method of Claim 4 wherein said
2 aminopolycarboxylic acids are ethylenediamine-
3 tetraacetic acid, propylenediaminetetraacetic acid or
4 mixtures thereof.
 - 1 6. The method of Claim 4 wherein said ferric
2 chelates are in an aqueous solution.
 - 1 7. The method of Claim 6 wherein said aqueous
2 solution further contains other carbon and/or nitrogen
3 sources.
 - 1 8. The method of Claim 7 wherein said other carbon
2 and/or nitrogen sources are sucrose and/or yeast
3 extract.
 - 1 9. The method of Claim 6 wherein the aqueous
2 solution is maintained at a pH of about 8.0 or below.
-

1 10. The method of Claim 9 wherein basal medium is
2 added to the aqueous solution.

1 12. The method of Claim 9 wherein a buffer is
2 added to the aqueous solution.

1 13. The method of Claim 12 wherein the buffer is a
2 phosphate buffer.

1 14. The method of Claim 9 wherein an acid is added
2 to the aqueous solution in amounts sufficient to
3 maintain the pH at about 8.0 or less.

1 15. The process of Claim 14 wherein the acid is an
2 inorganic mineral acid.

1 16. A method for the removal of metal chelates of
2 aminopolycarboxylic acid from an aqueous solution
3 containing such chelates wherein said metal is any
4 metal cation, other than Fe^{+3} , capable of chelating
5 with said aminopolycarboxylic acid which method
6 comprises:
7 a) adding a sufficient amount of a ferric salt to
8 the aqueous solution containing said metal chelates so
9 that a substantial portion of said metal chelates
10 become ferric chelates; and
11 b) adding Agrobacterium sp. (ATCC No. 55002) to
12 said solution while maintaining the pH of said solution
13 at about 8 or less.

- 1 17. A method of recovering iron from aqueous
 - 2 solutions containing ferric chelates of an aminopoly-
 - 3 carboxylic acid which method comprises
 - 4 a) adding a biologically pure culture of
 - 5 Agrobacterium sp. to an aqueous solution containing one
 - 6 or more ferric chelates of aminopolycarboxylic acid;
 - 7 b) maintaining the aqueous solution at pH of about
 - 8 8 or less at a temperature and for a period of time
 - 9 sufficient for iron to precipitate from solution; and
 - 10 c) recovering said iron.
-

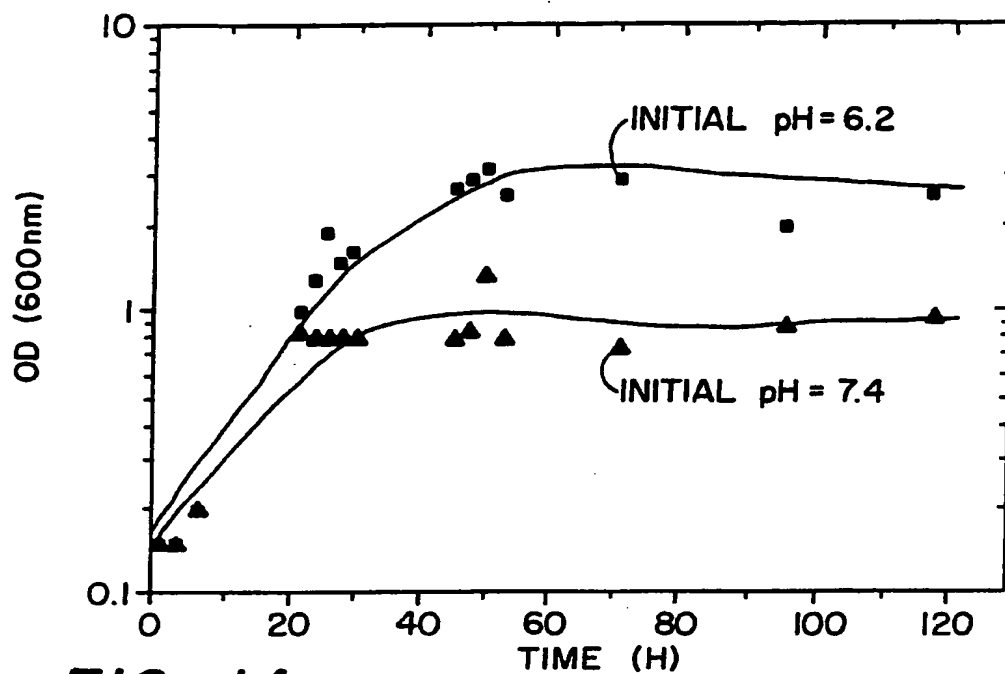


FIG. 1A.

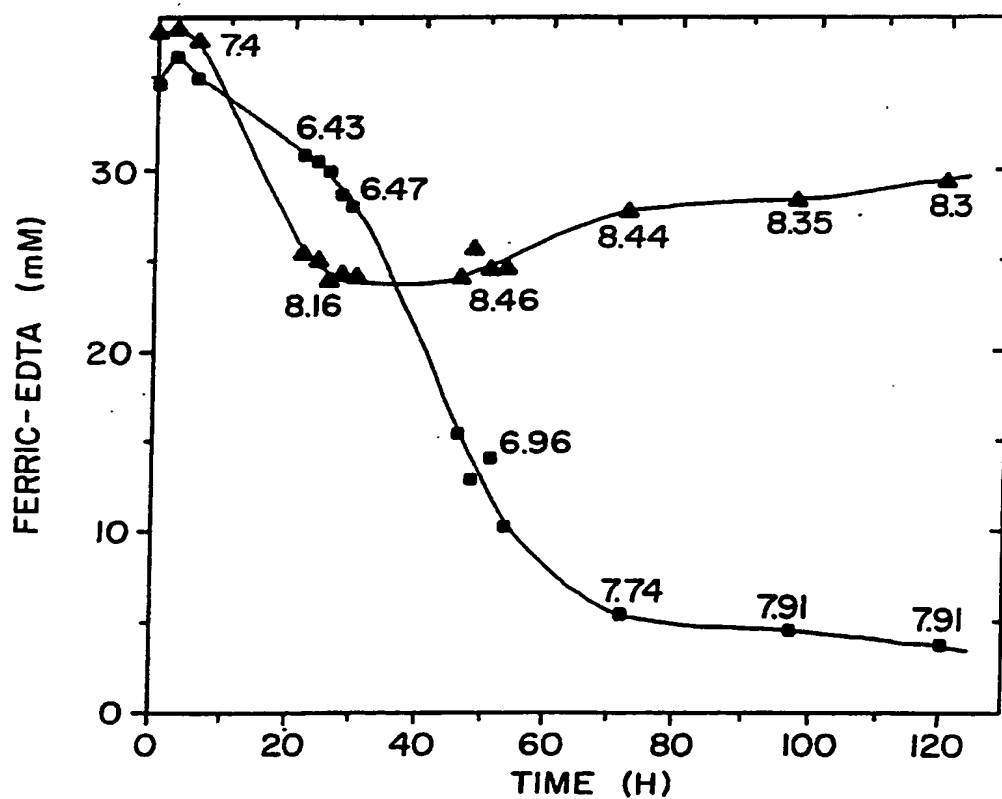


FIG. 1B.

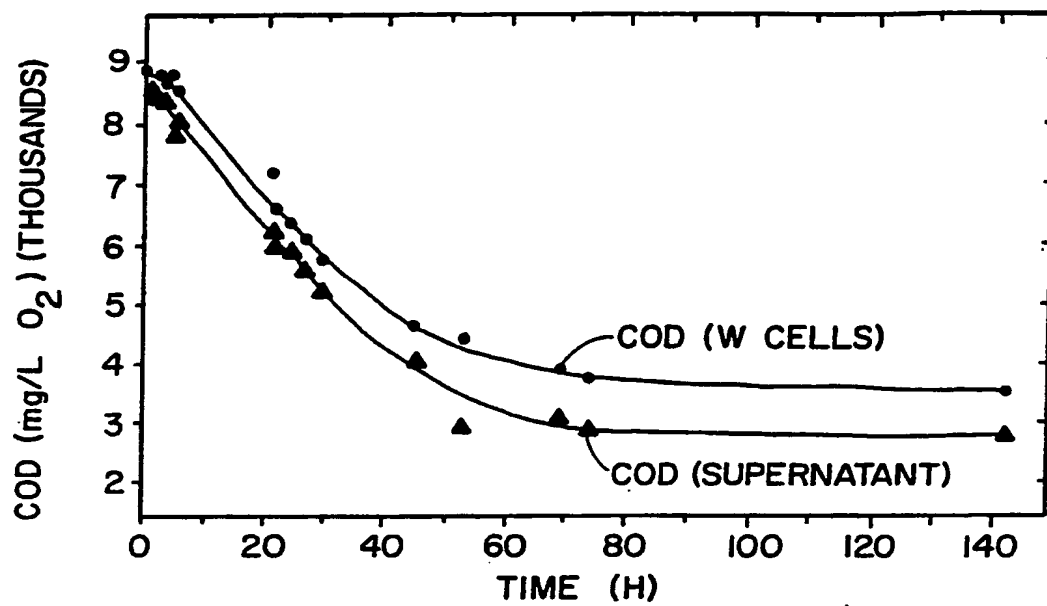


FIG. 2A.

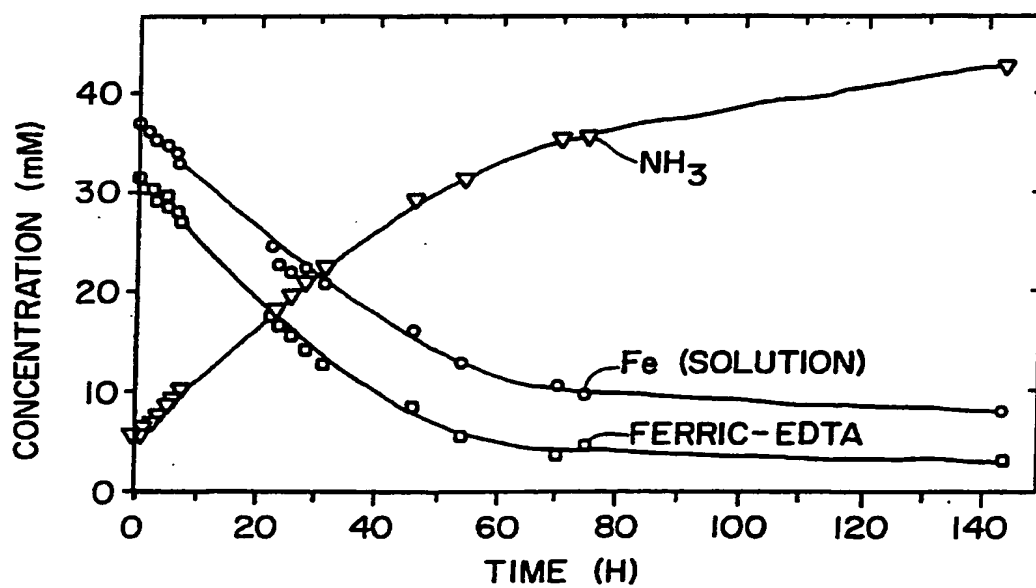


FIG. 2B.

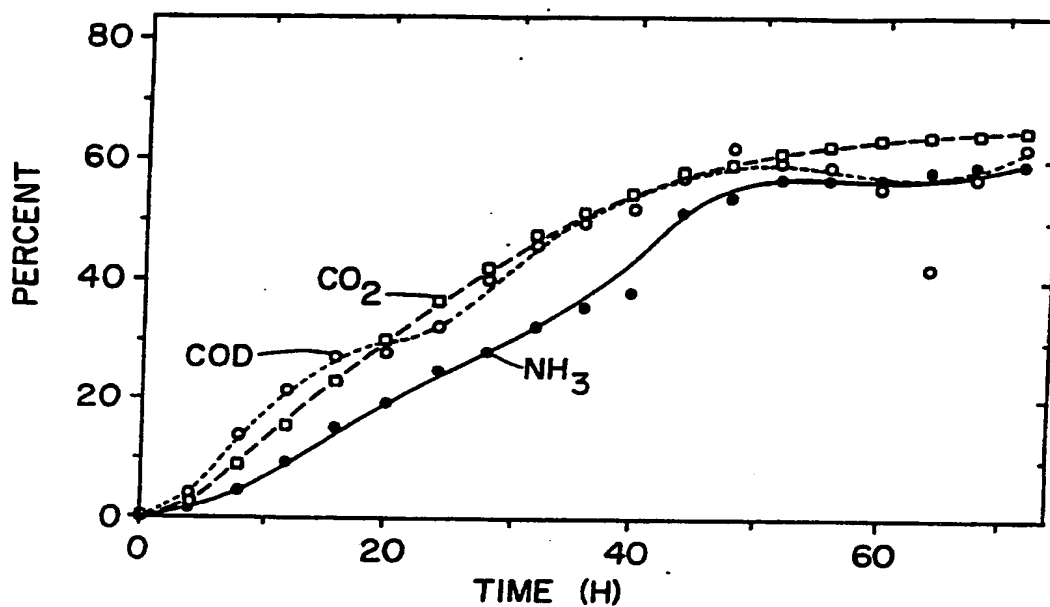


FIG. 3A.

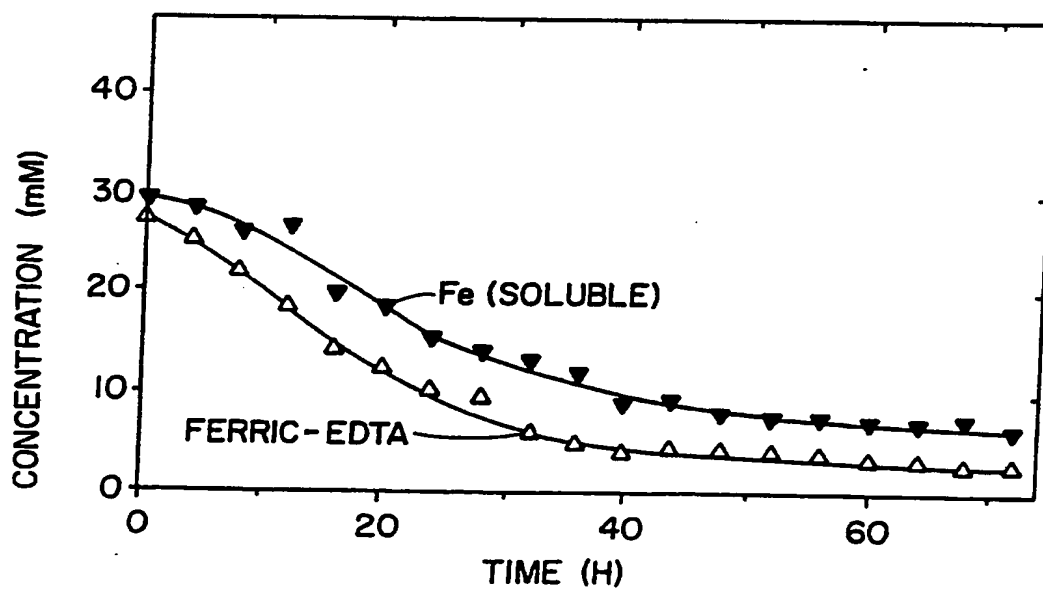


FIG. 3B.

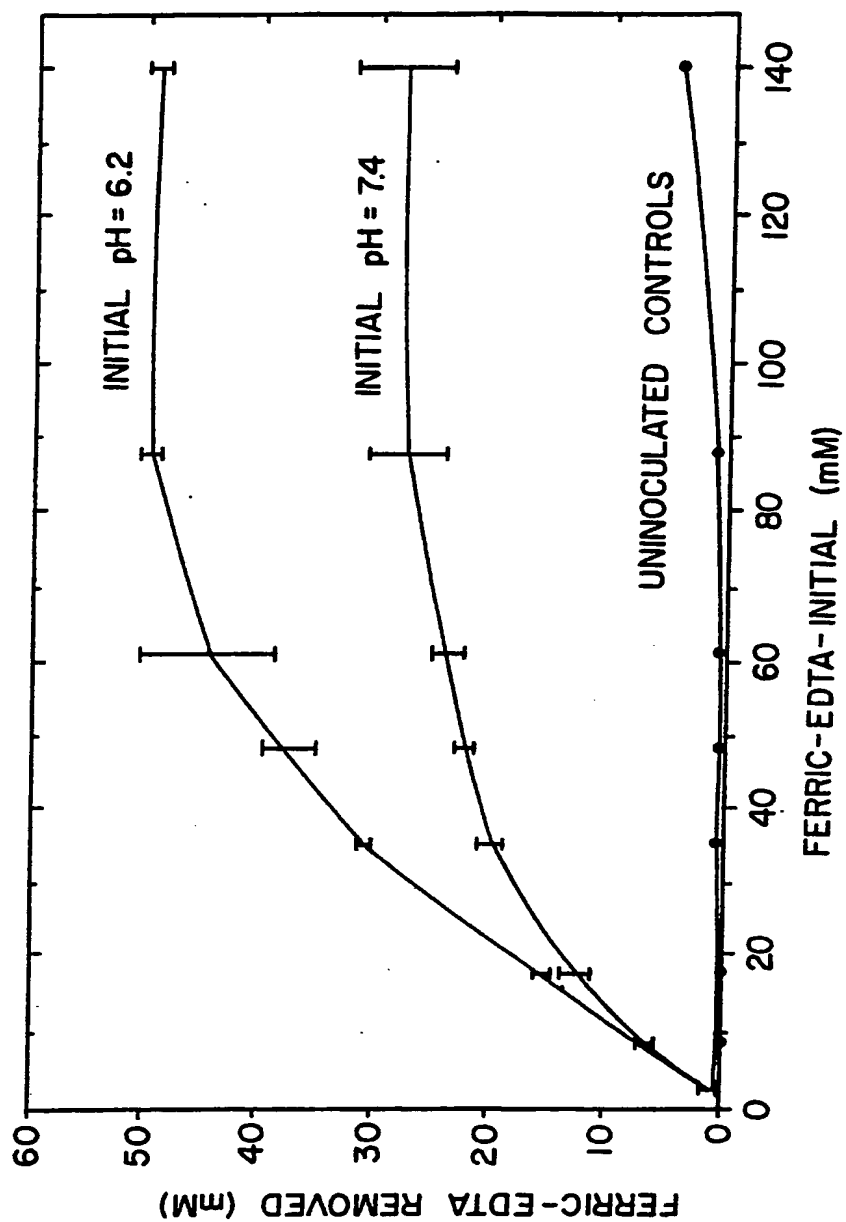


FIG. 4.

9683-106 (TSB)
Pt/Pd SHADOW (14 DEGREES)
1 μ m

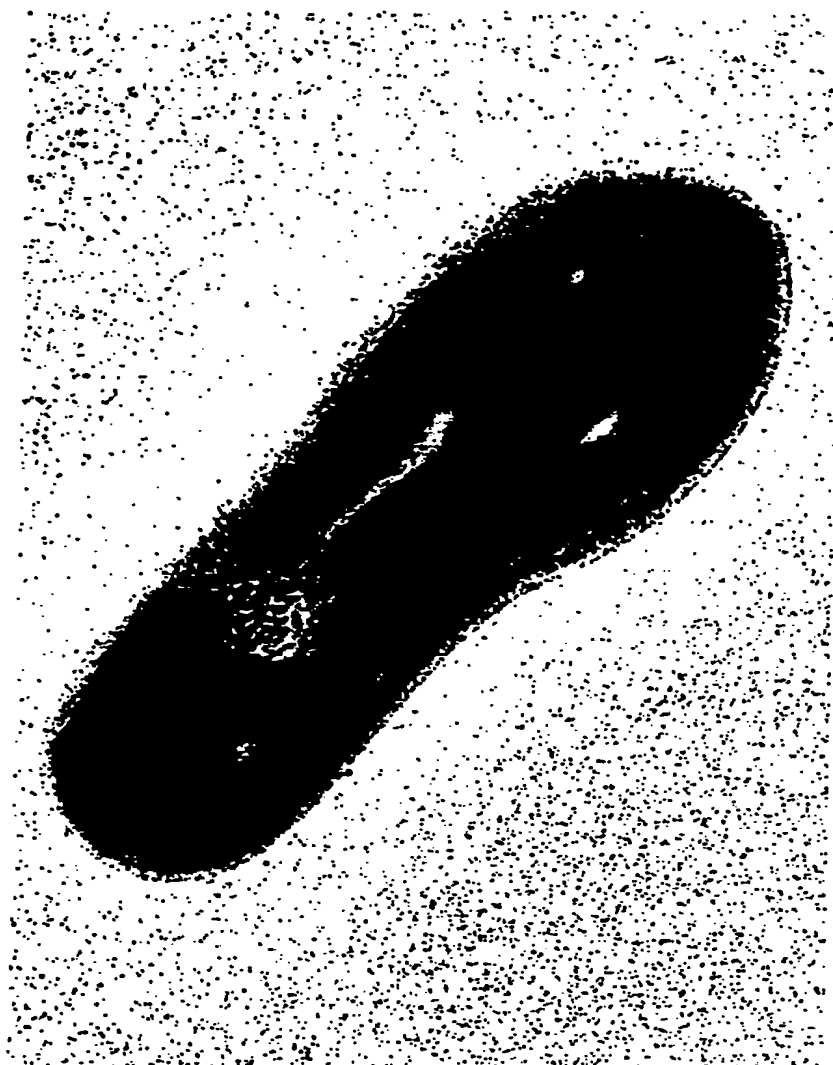


FIG. 5

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9683-106 (FeEDTA)
Pt/Pd SHADOW (14 DEGREES)

1 μ m

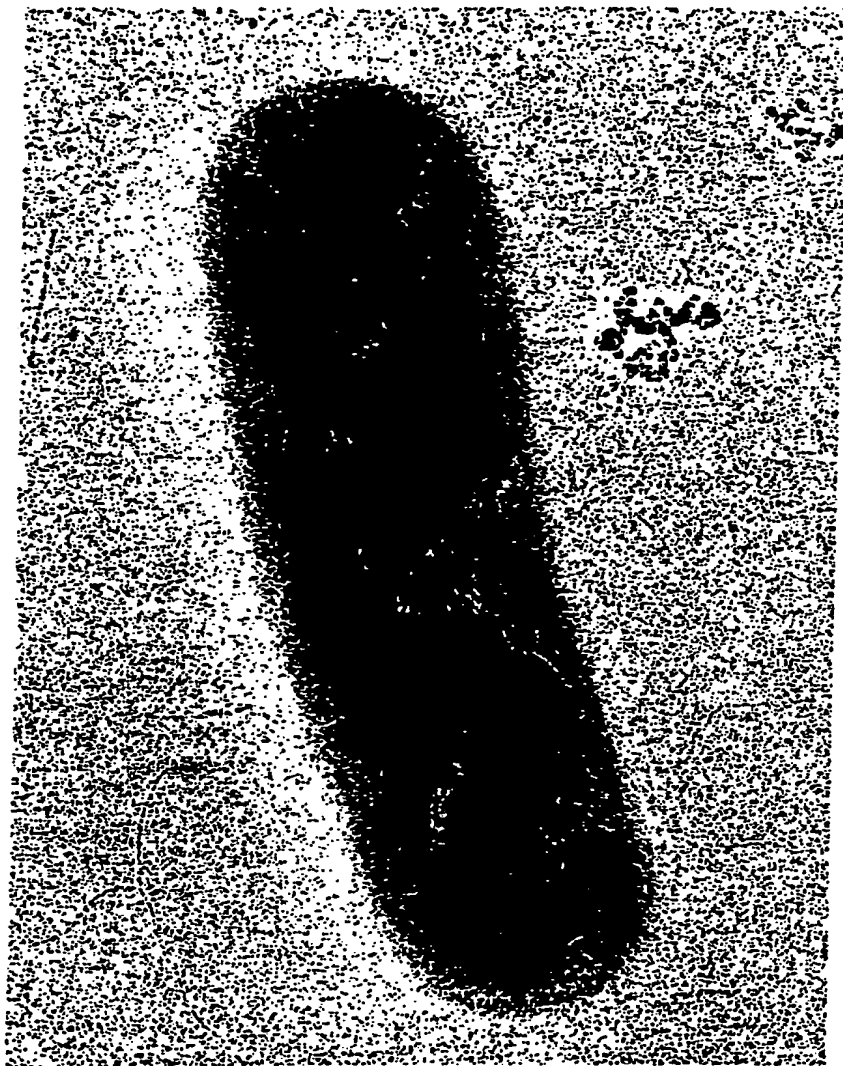


FIG._6

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02401

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC U.S. CL.: 435/252.2, 829, 262, 131, 168 IPC(5): C12P 9/00, 3/00; C12N 1/20		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	435/131, 168, 252.2, 262, 829	
Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched *		
CAS Online, Biosis		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X Y	JP, A, 5,843,782 (IMAI ET AL.) 14 March 1983, see entire document.	1-9, 12-15 10, 16-17
X Y	US, A, 4,673,644 (HARADA ET AL.) 16 June 1987, see entire document.	1-3 1-3
X Y	US, A, 4,634,667 (LINTON ET AL.) 06 January 1987 see entire document.	1-3 1-3
Y	Applied Microbiology, Volume 29, No. 6, issued June 1975, BELLY ET AL., "Degradation of Ethyl-enediaminetetraacetic Acid by Microbial Populations from an Aerated Lagoon", pages 787-794, see entire document.	10 and 16-17
A	BUCHANAN ET AL., "Bergey's Manual of Determinative Bacteriology", published 1974 by Williams & Wilkins (Baltimore, MD), see pages 264 and 265.	1-3
<p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claims or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
29 May 1991		15 JUL 1991
International Searching Authority		Signature of Authorized Officer
ISA/US		C.M. Geckle (vsh)